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Physiochemical Food System Studies
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No restrictions requested for this report.

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I. GENERAL

This report concentrates on two aspects of the research: the chemistry of formose sugar mixtures and the food utility of certain formose mixtures.

During this quarter five studies of various effects of formose diets have been finished or initiated. One, concerning the effects of HCHO, was partially reported in the annual report dated 22 February 1970. Some data from these studies is discussed in the section on food utility. The remainder of this data will be considered in the next quarterly report.

The report on the chemistry of formose mixtures is a summary of work performed in this area during the previous eight months. A number of small experiments on analysis and analysis techniques are discussed, and future directions are outlined.

A measure of "food utility" has been developed, which is discussed in detail. Values for food utility are presented for most feeding studies to date, and are discussed.

An effect on weight gain of control rats has been noted which is related to the date of birth. This is discussed.

II. CHEMISTRY OF FORMOSE: SUMMARY

During the last eight months, the chemistry of formose has been examined, in order to determine the most expedient methods of analysis and the chemical constitution of the mixture.

A complete discussion of the work is presented in Appendix A; highlights are presented here.

As previously reported, the formose mixture contains many compnents,, most of which appear to be sugars and/or polyols. Gas-liquid chromatography (GLC) is a standard technique of analysis of mixtures of chemically similar compounds. GLC techniques have been improved, although the principal technique still reduces all sugars to the corresponding polyols. No definitive data on the complete composition of formose is yet available.

Formaldehyde (HCHO) is the starting material for formose synthesis. Data is presented on the HCHO content of several formose mixtures. Also, techniques of HCHO removal are discussed. These data are of importance because HCHO is deleterious to most life forms, and formose cannot successfully be used as a food product if it contains appreciable quantities of HCHO.

In order to simplify formose analysis, several possible fractionations have been attempted, and are reported. Static reverse osmosis through microporus glass capillaries did not produce a separation. Nor did Ethanol-ether solubility techniques. On the other hand, various ion exchange techniques produced some separations, but not those sought.

A schema for separation of formose on the basis of functional groups is presented. Some results of experiments along this line are discussed. This schema will form the basis of future work.

In general, it must be reported that the formose mixture is quite difficult to analyze. Yet the techniques developed thus far are quite useful for comparative purposes. Also, techniques are being developed for analysis of certain fractions expected in formose.

A detailed experimental report of these data has been submitted to Dr. Jacob Shapira, the technical monitor. It is available from this laboratory on request.

III. FOOD UTILITY OF SOME FORMOSE MIXTURES

Two indices of "food utility" have been developed. They are presented in detail in Appendix B. It is prudent at this point to reiterate several points about these indices, FUC-1 and FUC-2.

The two indices, though similar, are not equal. Separate indices were developed to treat data of differing levels of detail.

A number of assumptions are implicit in the model as it is presently constituted. They are specified in Appendix B.

These measures operationally define "food utility", and may not correlate with other measures. Food utility is, in general, a measure of how "good" a substance is as a food. Yet "goodness" is not a quality which has been unanimously agreed upon by researchers -- it may be undefinable! Thus an operational definition must be used.

With these limitations, FUC-1 and FUC-2 are useful in this research. In particular, they may be used to compare among various experimental food treatments in which the appropriate experimental variables are held constant.

As outlined in the annual report of 22 February 1970, the feeding experiments in this research have followed a constant general pattern. Thus, it is felt that results between experiments in this work are comparable. Experiment 11 and subsequent ones have followed precisely the protocol presented as Appendix C.

Calculations of FUC-1 and FUC-2 for various experiments are presented in Tables 1 through 4. The relationship of FUC-1 to FUC-2 is discussed in the Appendix; data follow the theoretical relationship to a high degree.

Table 2 is a continuation of Table 1, presenting additional data. From these tables it is evident that formose LBM-3 and formose LBM-4 are quite similar in effect when treatment is made as defined. The low value for 5% LBM-3 in group 8-3 is considered to be spurious: in one 3-day period during experiment 8, animals exhausted their liquid supply.

Formose LBM-5 and LBM-6, tested at the 5% and 20% levels, showed low food utility. This was expected, due to the high levels of HCHO in these materials (Appendix A, p. 2). Linear correlations were performed between HCHO content (LBM-3 value was the mean of four values) and FUC-1 at each level. The 5% treatment showed r = -0.999; the 20% treatement showed r = -0.186.

Experiment 14 (to be reported fully in the next report) included HCHO treatments at various levels:

HCHO %	FUC-1
0.03125	1.0159
0.0625	1.0671
0.125	0.9776
0.25	0.7843

The linear correlation for these data is r =-0.945.

All of these data show the deleterious effect of HCHO on the weight gain of rats, not an unusual finding. However, preliminary analysis indicates that HCHO is definitely not the only contributor to the poor food utility of formose. A subsequent report will consider this relationship in more detail.

Table 3 and Figure 1 consider the food utility of various levels of formose LBM-3 and LBM-4. Although the experimental groups did not contain equal numbers of animals, the similarity of values for treatments with these two formose mixtures was such that it was considered useful to

mean the FUC-1 values at equivalent levels of treatment. This similarity is shown in Table 1 and Table 4.

In Figure 1, the value used for 0% formose is the theoretical value of 1. The data for 10 control animals in experiment 12 was randomly divided into two sub-groups of five animals, and FUC calculated for one sub-group using the other as control: FUC-1 = 0.9975, FUC-2 = 0.9927. This supports use of the theoretical value.

The relationship of food utility to dose, as shown in Figure 1, is that of the classic dose-response curve. However, low doses of formose produce FUC > 1. This is presently considered a technique artifact. As previously reported (Chermside et al, "Some Aspects of the Toxicity of Formose Sugars" Proc. West. Pharmacol. Soc. 14, Appendix C to annual report of 22 February 1970), low doses of formose increase the liquid intake of rats. This probably results in overall retention of fluids by the animal, producing increased weight. At present it is not deemed necessary to confirm this explanation experimentally.

From all of these data, several conclusions are drawn:

1) The food utility indices FUC-1 and FUC-2 are useful measures for this work.
2) Formose LBM-3 and LBM-4 are similar in effect, and much less deleterious than LBM-5 and LBM-6.
3) HCHO contributes to the deleterious effects of LBM-5 and LBM-6.
4) Other formulations of formose can be tested and compared to these.

Finally, it is considered possible that food utility can be correlated with the constitution of differing formose mixtures and fractions by use of FUC-1 or FUC-2 in analyzing feeding studies. Therefore, work will continue on fractionation and analysis of formose.

Table 1

Food Utility Constant: Values for Formose Treatments

Treatment	Group	FUC-1	FUC-2
5% C	1 - 1	0.6157	-0.0616
5% LBM-3	2 - 3	0.7895	0.3898
5% LBM-3	7 - 2	0.7374	0.1447
5% LBM-3	8 - 3	0.5212	-0.5454
5% LBM-3	10 - 2	0.7310	0.2018
5% LBM-4	10 - 3	0.7325	0.2064
5% LBM-4	14 - 2	0.7922	0.3401
5% LBM-4	15 - 2	0.7463	0.2031
5% LBM-4	17 - 3	0.7903	0.3449
5% LBM-5	15 - 3	0.3235	-1.1250
5% LBM-6	15 - 4	0.2215	-1.4453
10% LBM-3	7 - 3	0.2422	-1.4684
10% LBM-3	14 - 4	0.2602	-1.3502
10% LBM-4	10 - 4	0.2626	-1.1880
10% LBM-4	14 - 3	0.2442	-1.4010
10% LBM-4	17 - 6	0.1875	-1.5387
20% LBM-3	7 - 4	0.1129	-1.8894
20% LBM-4	10 - 5	0.1389	-1.5550
20% LBM-4	11 - 2	0.1380	-1.6794
20% LBM-4	12 - 2		
20% LBM-4	13 - 2	0.0645	-1.6917
20% LBM-4	15 - 5	0.0599	-1.9531
20% LBM-4	17 - 7	0.0597	-1.9379
20% LBM-5	15 - 6	0.2315	-1.4140
20% LBM-6	15 - 7	-0.0271	-2.2265

Table 2

Food Utility Constant:

Additional Values for Formose

Treatment	Group	FUC-1	FUC-2
2.5% LBM-3	2 - 2	1.0347	1.1007
2.5% LBM-4	17 - 2	1.0148	1.0465
6.5% LBM-4	17 - 4	0.4753	-0.6395
8.5% LBM-4	17 - 5	0.3066	-1.1666
40% LBM-3	7 - 5	0.1371	-1.8105

Table 3

FUC-1 Mean Values, Formose LBM-3 and LBM-4, Varying Levels

Level	# Values	FUC-1	s.d.
2.5%	2	1.0248	0.0138
5%	7	0.7599	0.0293
6.5%	1	0.4753	--
8.5%	1	0.3066	--
10%	5	0.2393	0.0303
20%	6	0.0957	0.0387
40%	1	0.1371	--

Table 4

Mean Values of FUC-1, Formose Treatments

Treatment	# Values	Mean Value	s.d.
5% C	1	0.6157	--
5% LBM-3 *	3	0.7526	0.0321
5% LBM-4	4	0.7653	0.0307
5% LBM-5	1	0.3235	--
5% LBM-6	1	0.2215	--
10% LBM-3	2	0.2512	0.0126
10% LBM-4	3	0.2314	0.0392
20% LBM-3	1	0.1129	--
20% LBM-4	5	0.0922	0.0422
20% LBM-5	1	0.2315	--
20% LBM-6	1	-0.0271	--

* one value excluded, judged to be influenced by inaccurate experimental technique (see text, p. 4).

Including this value, N = 4, Mean FUC-1 = 0.6948, s.d. = 0.1187

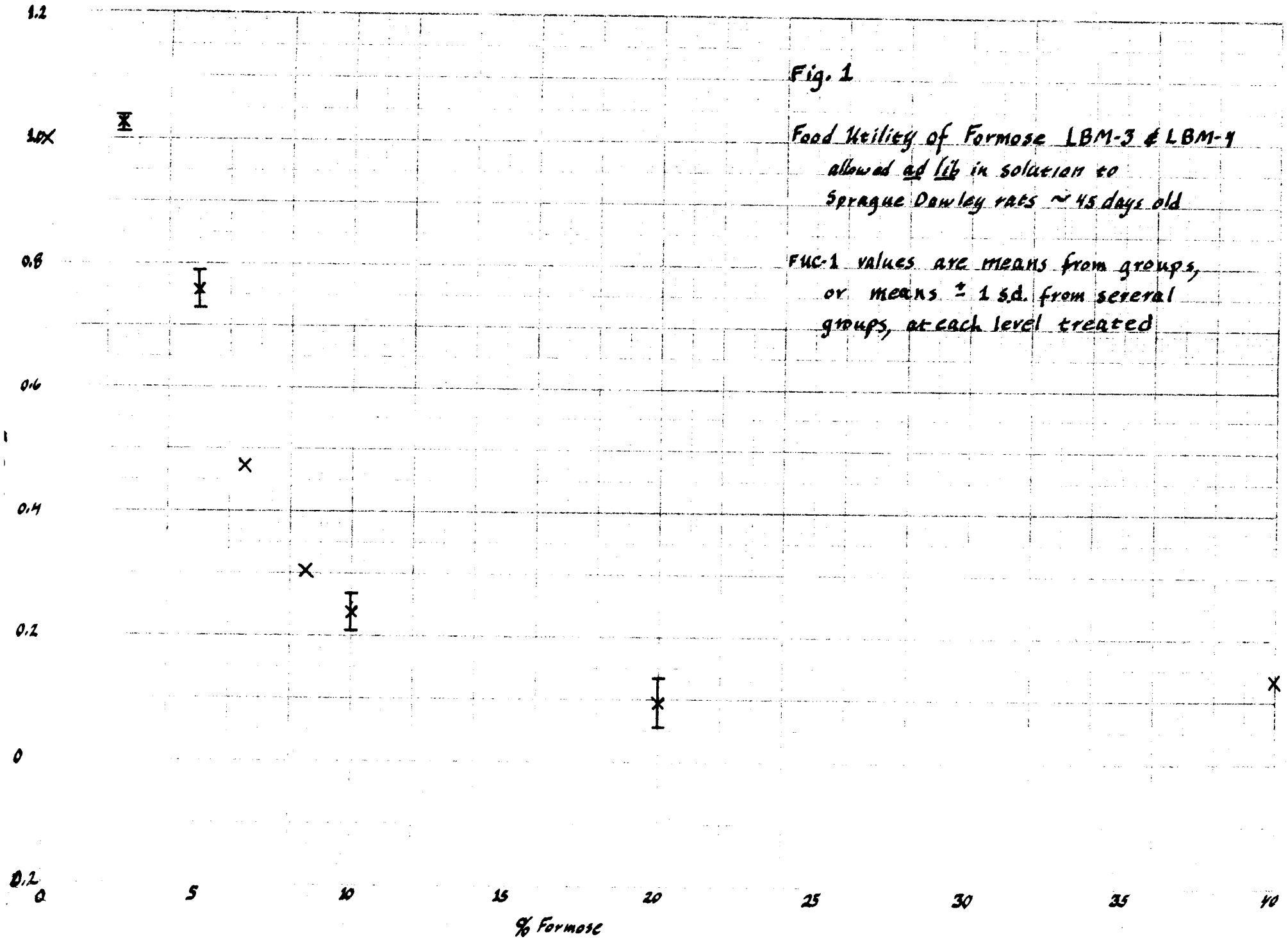


Fig. 1

Food Utility of Formose LBM-3 & LBM-4 allowed ad lib in solution to Sprague Dawley rats ~45 days old

FUC-1 values are means from groups, or means ± 1 s.d. from several groups, at each level treated

IV. Effects of Date of Birth on Weight of Control Rats

The Sprague-Dawley rats used in these experiments are a random bred strain. Such a strain has been known to grow at varying rates, dependant on the season of birth. This effect, if it exists, could affect the reliability of inter-experimental comparisons of results. Therefore an estimate of the effect was sought.

Animals from a single supplier and colony (A.J. Schmidt, Berkeley Colony) have been used in eight experiments over a 7 month period. Control groups are randomly selected from animals received, and are treated uniformly. The animals are received the same day they are shipped, and upon receipt are weighed and individually caged. They are allowed ad lib chow and water, and are handled 3 to 7 times weekly thereafter, throughout the experiment.

Table 5 summarizes the data examined. The individual weights were analyzed by analysis of variance for the effect of groups identity on weight observed at 46 days of age. One group was measured at 47 days, but in the light of the magnitude of the effect, this was judged not to affect the results.

The F ratio computed was 5.639, with d.f. of 7 and 33. This is highly significant ($F_{.001} = 5.12$, d.f. = 6, 30). It is concluded that the identity of the group has a significant effect ($P < 0.001$) on the weight at a given age of control animals in these experiments. Date of birth is considered to be the principal variable in group identity. The data accumulated is presently insufficient to identify seasonal or annual cycles.

Table 5

Group Mean Weights, Age 46 Days

Expt. #	birth date	age	N	mean wt, g	s.d.
8	7/22/69	46	4	200.3	24.5
10	9/3/69	47	5	189.6	21.6
11	10/6/69	46	2	153.5	2.1
12	10/20/69	46	10	175.5	13.6
13	11/3/69	46	5	196.2	5.1
14	11/24/69	46	5	156.4	13.7
15	1/19/70	46	5	167.6	14.8
17	2/16/70	46	5	185.8	7.1

Male Sprague-Dawley rats obtained from A.J. Schmidt, Berkeley, colony at age 35 ± 5 days, individually caged and fed ad lib chow and water through measurement period.

Appendix A

Chemistry of Formose -- Discussion

Thomas Putkey, Ph.D.

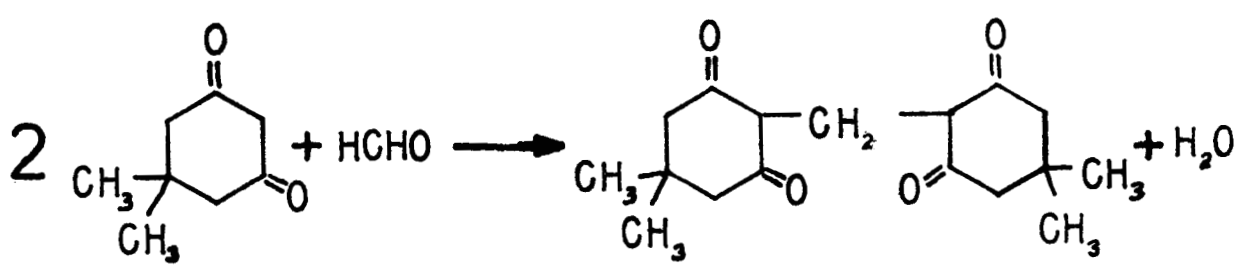
Chemistry of Formose - Discussion

Studies of the chemical composition of the formose sugar melange were initiated so as to gain insight into factors affecting the food utility of this material. The following areas have been considered and are discussed here: whole analysis - formaldehyde content, formaldehyde removal, improving GLC analysis; simplified analysis - simpler formose, physical fractionations, chemical fractionations; future directions.

Formaldehyde Content

A study of the chemical composition of the formose sugars was initiated so as to gain insight into the factors affecting the food utility of these sugars. The first aspect of the composition of the formose mixture to be looked at was the amount of formaldehyde, either free or bound, in the formose sugars, and how it might be removed.

The method used to analyze for the formaldehyde content of the formose mixture was the gravimetric method which is known to be quantitative for formaldehyde (1), dimedon (I) and formaldehyde (II) in solution reacting to form insoluble methylene bis-methone (III).



I II III

The following formaldehyde values were obtained for different batches of formose:

Batch	% Formaldehyde
formose C	trace
LBM-3 (shelf)	2.3
LBM-3 (from Chermside)	0.5
LBM-3A	0.49
LBM-3B	2.3
LBM-4	1.1
LBM-5	7.0
LBM-6	8.8

In addition to the free formaldehyde in the formose sugar melange, there may be formaldehyde bound to the formose sugars themselves, as formaldehyde has been reported to complex with certain sugars (2). There should be no formals or hemi-formals formed between formaldehyde and the formose sugars, as the sugars were produced under basic conditions, and formal formation requires acid catalysis. Treatment of the formose sugars with dilute acid would break any formaldehyde-sugar complexes, as well as formals or hemi-formals, shoudl they be present. The amount of bound formaldehyde could be determined from any increase in the amount of methylene bis-methone from the sugars treated with the acid greater than the amount of dimedon derivative obtained from the usual dimedon analysis of the formose sugars. The formaldehyde content of LBM-4 was found to be 1.1% by the usual dimedon method, and a formaldehyde content of 1.01% was found when 0.1 N hydrochloric acid solution was used in the analysis. The close agreement of these two analyses indicates that there was no significant amount of bound formaldehyde in the LBM-4.

Analysis of known formaldehyde solutions in 0.1 N hydrochloric acid and methanol solution showed that methylene bis-methone either would not form in 0.1 N hydrochloric acid solution or it was soluble at this acid concentration. So for the formaldehyde analyses to be quantitative, the acid solution had to be neutralized. Also, methylene bis-methone was found to be stable under the acid conditions of this analytical method.

Formaldehyde Removal

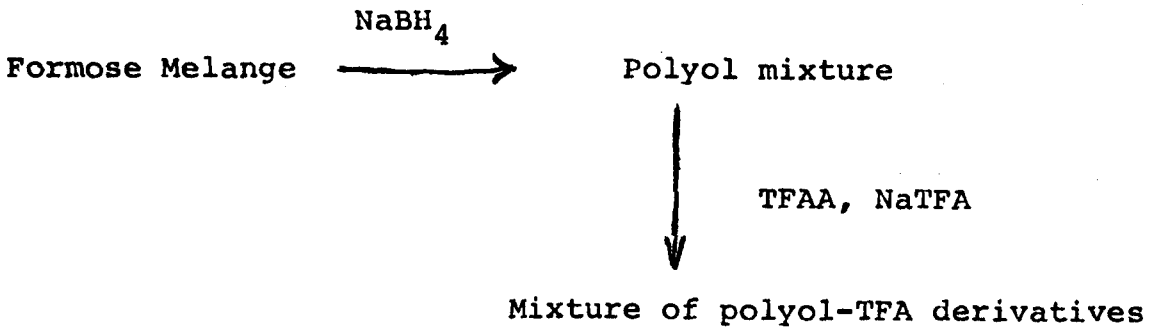
Once the formaldehyde in the formose sugars was determined to be free formaldehyde only, its removal was desired. This would eliminate formaldehyde as a cause of the deleterious effects of formose sugars observed when fed to rats. This requires a reasonably large scale process, as experiments with rats call for 500 g or more for each experiment. It seems most effective to remove formaldehyde from all formose immediately after synthesis. The first method attempted for the removal of the formaldehyde was to heat an aqueous solution of the formose sugars to 65° while nitrogen was bubbled through the solution. Some formaldehyde was removed in this manner, but the removal was so slow that this process was judged not feasible. Next, nitrogen was passed through a 50% LBM-4 solution in 0.1 N hydrochloric acid, as Dr. J. Schneider reported formaldehyde was removed rapidly from an acid solution of the formose sugars, at least on an anlytical scale, by this method (3). The formaldehyde content of the much larger amounts of formaldehyde solutions that we used was decreased, but not enough to make this process feasible.

A quantitative separation of formaldehyde from sugars by the use of Amberlite IRA 400 resin in the bisulfite form has been reported by Samuelson (4). Using his procedure, we were

able to remove quantitatively formaldehyde from an aqueous solution, and from a solution of formaldehyde, fructose, sucrose and mannose. This method was applied to a solution of LBM-4, and the formaldehyde was removed quantitatively without any effect on the sugars, but at the present time this method has been used only on analytical samples of LBM-4, and not on large amounts of the sugars.

Improving GLC Analysis

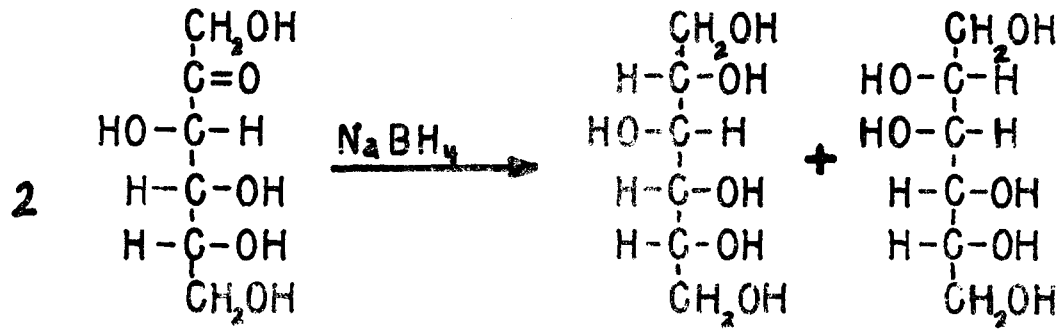
The analytical technique used to compare and identify components of formose sugar mixtures was that developed by Dr. J. Shapira and co-workers (5). Reduction of the formose sugar mixture with sodium borohydride ($NaBH_4$) was followed by dervitization of the resulting polyols with trifluoroacetic anhydride (TFAA) saturated with sodium trifluoroacetate.



The mixture of derivitized polyols was analyzed by gas liquid chromatography (GLC), and the chromatograms showed the presence of at least thirty-five components. A number of these components were identified by comparing the retention times of the trifluoroacetyl (TFA) derivatives of known polyols to the retention times of peaks in the formose mixture, and assuming that peaks of the same retention times were from the same polyol TFA derivative, and hence from the same polyol. The sugars which led to these polyols upon reduction were assumed to be present in the formose sugar mixture.

Some trouble has been encountered in this analytical method in that certain single ketoses and aldoses after reduction gave more than the theoretically expected number of peaks from the TFA derivatives of the polyols. The question as to whether these spurious peaks arose from the reduction reaction or from the derivitization process was investigated to improve the above analytical method.

The first concern was the faint possibility that there was a pH variable involved in the $NaBH_4$ reduction. The literature states that $NaBH_4$ decomposes rapidly in acid solution or in a solution containing a better proton donor than water (6), that fructose will isomerize to form some glucose in alkaline solution (7), and that $NaBH_4$ reduction of fructose (V) has been found to give equal amounts of sorbitol (V) and mannitol (IV) (8).



IV V VI

The sodium borohydride reduction of ketoses was considered first. The reduction of fructose was carried out under various pH's from 4 to 11, in order to determine if the reduction was dependent upon the pH of the fructose solution. This was done in several buffered solutions, in water, and in sodium hydroxide solutions. The polyols from the reduction were converted to their trifluoroacetyl

derivatives, and the mixtures were analyzed by gas chromatography. All chromatograms showed two peaks, one corresponding to sorbitol and one corresponding to mannitol, in approximately equal amounts. The final pH of each reduction mixture was about 11, demonstrating adequate borohydride present to react first with all the acid and then to reduce the fructose. The reduction mixtures would be expected to have about the same final pH, as the metaborate ion from the borohydride decomposition would act as a buffering agent to make the final pH values approximately equal (9). As the borohydride reduction of carbonyl groups is rather well documented in the literature, it was decided to accept this demonstration of pH independence in the $NaBH_4$ reduction of fructose, and turn to the reduction of the formose sugars.

Samples of LBM-4 formose were reduced with varying amounts of $NaBH_4$ including a large excess in order to ensure that all the sugars in the formose melange were reduced completely. The GLC chromatograms of the TFA derivatives of the polyol mixtures from the various runs were the same, indicating adequate $NaBH_4$ to reduce all sugars in all reductions attempted. No further work was considered necessary, once it was demonstrated that sufficient borohydride had been used.

As it was most unlikely that the reduction of the sugars was giving rise to undesired peaks in the GLC analysis of the TFA derivatives of the polyols from the reduction of sugars, attention was turned to the TFA derivatization process, as it could well be that undesired reactions during the derivatization process were forming compounds which gave rise to the unexpected peaks.

The derivitization process was carried out on known polyols in the following solutions: trifluoroacetic anhydride (TFAA) saturated with sodium trifluoroacetate [method of Shapira (5)], TFAA and acetonitrile with sodium trifluoroacetate [method of Vilkas (10)], TFAA and tetrahydrofuran [method of Tamura and Inamari (11)], TFAA and acetonitrile, and TFAA and tetrahydrofuran containing sodium trifluoroacetate. The simplest gas chromatograms were obtained using the TFAA and acetonitrile solution with sodium trifluoroacetate. The TFA derivatives were found too stable for long periods in this solution. In addition, several sugars gave only the expected number of peaks when derivitized in this solution, as had not been the case in TFAA saturated with sodium trifluoroacetate and TFAA and acetonitrile solution. The chromatograms of the TFA derivatives formed in the solutions containing tetrahydrofuran showed two spurious peaks which increased in height with time. Also, the TFA derivatives were unstable in these solutions, as Tamura and Imanari had reported. On the basis of the above results, the TFAA and acetonitrile solution containing sodium trifluoroacetate was chosen as the solution for forming the TFA derivatives of polyols in the remainder of this report.

Simplified Analysis - Rationale

Up to this point in the investigation, the principal technique for identifying the many components of the formose melange was GLC of reduced mixtures. However, this method of analysis has serious drawbacks which makes it of dubious value for more than a partial definition of the composition of the formose sugars.

First, the assumption is made that peaks of a given retention time represent identical compounds. There have been some exceptions to this in our work. In such a com-

plex mixture as formose, identity of compounds from equal retention times should be verified by other methods, such as mass spectroscopy, or nuclear magnetic resonance. Further, each peak in the chromatogram of the formose mixture may arise from more than one substance, and this cannot be known by gas chromatography on only one column.

The mixture of TFA derivatives analyzed by GLC is one of polyols from the reduced formose sugars, and not the formose mixture itself. There are polyols present in the formose mixture before reduction, and these cannot be distinguished from the polyols from the reduction of aldoses, ketoses, or aldonic acid lactones. Also, a single polyol may arise from several sugars, and it would not be possible in many cases to determine which of several parent sugars gave rise to this polyol. Finally, it would be necessary to have a great number of known polyol standards in order to compare the retention times of their TFA derivatives to the retention times of the peaks of the TFA derivatives of the polyols obtained from the formose sugars. Many of these polyols would have to be synthesized, which would be a task of no small magnitude. In view of these difficulties, it was decided to seek other methods of identifying the compounds of formose sugar mixtures, while at the same time utilizing the information of the composition of these mixtures gained from the GLC analysis of the TFA derivatives of the reduced formose mixtures.

Synthesis of Simplified Formose

One obvious means of simplifying the identification of the formose sugars would be to produce a simpler formose mixture. Dr. Shapira had information that the barium

hydroxide [$Ba(OH)_2$] catalyzed condensation of formaldehyde gave a relatively simple mixture of sugars. So we made a synthesis of formose sugars in the flow reactor[18], using $Ba(OH)_2$ as the catalyst, but the mixture of sugars produced was little different from the formose sugars produced with calcium hydroxide [$Ca(OH)_2$] the usual catalyst. A small amount of formose sugars was formed by the batch method using $Ba(OH)_2$ as the catalyst, but this mixture, also, was as complex as that from $Ca(OH)_2$ or the flow reactor. No further attempts have yet been made to produce a simpler mixture of formose sugars.

Fractionation of Formose - Rationnale

If the rather complex mixture of formose sugars could be separated into fractions of a lesser number of components, it should be easier to identify the components of the simpler mixtures. Separation of the formose sugars into less complex mixtures was undertaken by both physical and chemical methods.

Reverse Osmosis

The physical method of separation attempted was that of static reverse osmosis[21], and these experiments were carried out with the kind assistance of Dr. Ted Wydeven of Ames Laboratories. The basic principle of the reverse osmosis procedure is to place a solution around a microporuous glass capillary, and to place a high pressure on the liquid to force it through the pores of the capillary. Solute molecules in the liquid may be rejected by the pores of the capillary, i.e., not pass through the pores, or they may pass through it at a much slower rate than the solvent molecules. It was hoped that the smaller molecules in an aqueous solution of the formose sugars would pass through the pores much faster than the larger molecules, or that there would be a cut-off point where only molecules up to a certain size would

would pass through the pores, and molecules larger than these would be rejected selectively. The results of the reverse osmosis experiments showed that there was a high overall rejection of the sugars by the capillaries, but that there was no selective rejection, i.e., the composition of the formose sugars that came through the pores was the same as that of the feed solution. Experiments on a known solution of glyceraldehyde, xylose, and glucose showed that there was a high total rejection on these sugars, but that there was no selective rejection. No further reverse osmosis experiments on sugar solutions were carried out, as the above results offered little hope of separation by this method.

Ethanol-Ether Solubility

Acherlof and Mitchell (13) report separation of formose into two fractions. A 25% solution of formose in 95% ethanol was treated with an equal volume of ether; approximately equal weights of soluble and insoluble materials were found. These fractions were reported to differ in the proportion of low molecular weight products contained.

This procedure was replicated using 20 g of LBM-4. The proportion of soluble to insoluble material was about 2:1. GLC of these samples showed no apparent difference of either from one another or from an untreated sample of LBM-4, in terms of content and distribution of reduced carbohydrates.

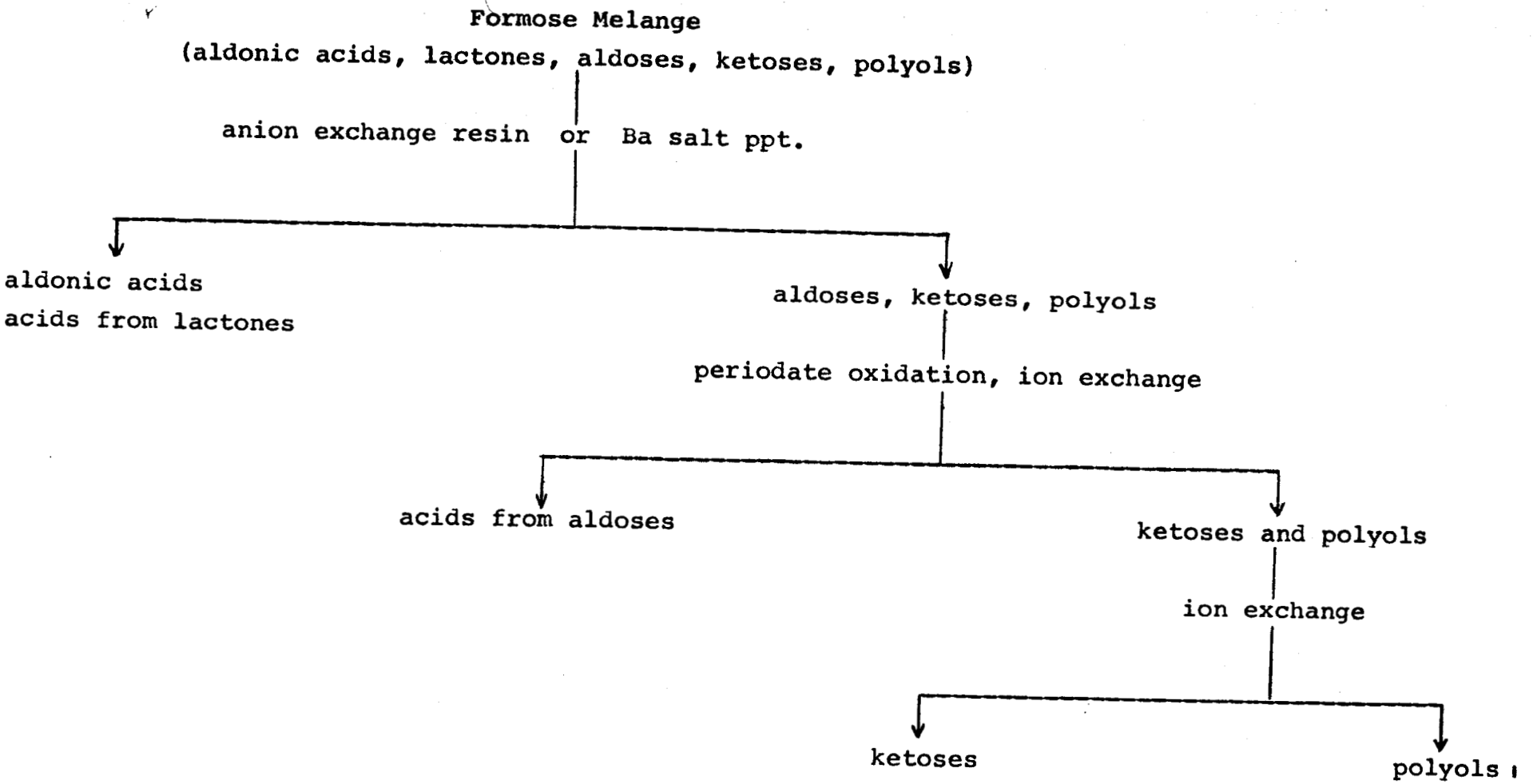
It is considered probable that the apparent separation is a matter of reduced solvability equally affecting all carbohydrates in the mixture. Feeding studies may, however, be made with similar fractions in lieu of a more precise determination of possible differences between fractions.

Separation of the Basis of Functional Groups

The formose sugars can be classified by functional groups into mixtures of aldonic acids, aldonic acid lactones, aldoses, ketoses, and polyols. A separation of these based on differing chemical properties of the functional groups would give sugar mixtures much simpler than the formose mixture, and hopefully easier to identify. It may be that a single sugar in formose could have more than one of the above mentioned functional group, such as an carboxylic acid and an aldehyde group on the same molecule, and this would have to be taken into account in any scheme of analysis for the identification of the aldonic acid mixture obtained.

A possible schema for chemical separation of the formose sugars is shown on the following page.

First, aldonic acids and lactones would be removed by ion exchange resins or by precipitating the barium salts of the aldonic acids from alkaline methanol solution. Then the aldoses in the remaining mixture would be oxidized with hypoiodite to the corresponding aldonic acids, and these acids would be separated from the ketoses and polyols, hopefully with ion exchange resins. The simpler mixtures thus obtained would require efforts to identify them varying in proportion to the complexity of these "simple" mixtures. Yet it would not be necessary to identify all components of these mixtures before using them for feeding studies. As each group of sugars of a particular functional group was removed, the remaining material could be tested for biological effects. Identification of the desireable or undesireable fractions could be undertaken; the identification of some fractions can be delayed until there be the time or need to do so. This approach would depend on the availability of a single large batch of formose, in order to assume consistent content over the analytical and feeding study program.



SEPARATION SCHEMA

Aldonic Acids and Lactone Separation Attempts

The ion exchange resin used in the treatment of formose sugars for the removal of aldonic acids and lactones was Amberlite IRA 400 in both the bicarbonate form and the free hydroxide form. They were prepared by washing the chloride form of the resin with sodium bicarbonate solution and sodium hydroxide solution respectively, according the the procedure of Samuelson (14). An aqueous solution of LBM-4 was placed on the resin in the bicarbonate form, and the resin was washed with water. All the LBM-4 was recovered unchanged from the water eluate. In order to remove any aicds from the resin, it was washed with hydrochloric acid solution. The acidic eluate was neutralized and the resulting solution was taken to dryness. No aldonic acids were found in the residue.

It was considered possible that the aldonic acids are too weak, as acids, to form their anions with the bicarbonate anion, so the resin in the free hydroxide form was used to remove any acids. When a solution of LBM-4 was passed rapidly through the resin (8 ml/min), only one-third of the sugars were found in the aqueous eluate. Washing the column with HCl solution yielded a syrupy material from the eluate. This material and the residue from the aqueous eluate were analyzed by GLC of the TFA derivatives of reduced polyols. The chromatogram obtained from the acid wash showed lesser amounts of 3- and 4-carbon sugars, and more material corresponding to substances beyond the 6-carbon sugars, when compared to the chromatogram of the residue from the aqueous wash.

When a solution of LBM-4 was passed through the resin in the hydroxide form at a slow rate (0.5 ml/min) only a trace of residue was obtained from the aqueous eluate. Washing the column with hydrochloric acid solution yielded a syrupy material which corresponded to 57% of the formose placed on the column. This material was analyzed as above, and the GLC chromatogram obtained showed peaks corresponding

to 3-, 4-, 5-, and 6-carbon sugars, and no intensification of the peaks beyond the region of the 6-carbon polyols.

It is evident that the amount of formose sugar that is retained on the resin varies with the flow rate over the resin column, and that sugars other than aldonic acids are being retained on the column. Hence this treatment of formose sugars cannot be used to remove aldonic acids and lactones. Furthermore, sugars have been known to isomerize, degrade, and oxidize on strongly basic resins (15), and these undesired reactions on the resin can add to the number of compounds present in the formose mixture. Thus it is not feasible to use strongly basic resins to separate or remove components from the formose in order to obtain mixtures for feeding studies or analysis.

Next, the separation of the aldonic acids and lactones was attempted by precipitating the barium salts of the acids from a basic methanolic solution of the formose sugars. The slight solubility of the barium salts of aldonic acids in methanol has been utilized in carbohydrate characterization procedures (16) and the precipitation of the barium salts is quantitative (16, 17). A solution of LBM-5 and barium iodide in methanol containing a small amount of water was made alkaline with methanolic potassium hydroxide, but only a trace of precipitate had formed after three days. A solution of LBM-6 in methanol-water was treated in the same manner, and again, only a trace of precipitate was formed.

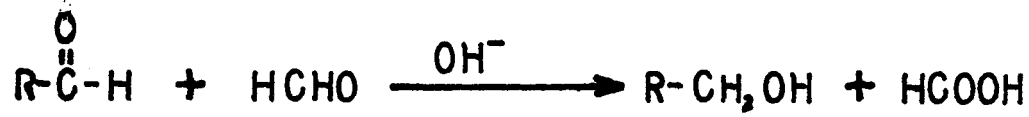
Aldonic Acids and Lactones in Formose Synthesis

The formose starting material used in these two experiments (LBM-5, LBM-6) had not been passed through the mixed-bed ion exchanger which is part of the usual formose synthesis procedure. This step was omitted in order to determine the amount of aldonic acid and lactone formation taking place in the synthesis itself.

Evidently there is no more than a small volume of aldonic acid and lactone formation during the synthesis of formose by the flow reactor technique (18). If lactones were present, they would be expected to open and to precipitate as the barium salts of the acids, in the above reactions.

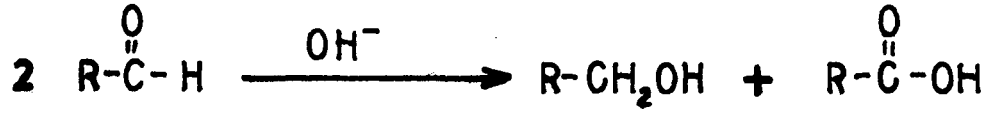
This result is not unreasonable, as the cross-Cannizzaro reaction between formaldehyde (II) and an aldose (VII) in the formose mixture would yield the polyol (VIII) from the reduction of the aldose, and formic acid (IX) from the oxidation of formaldehyde, as formaldehyde is known to reduce aldehydes to alcohols while it is oxidized to formic aicd (16). The Cannizzaro reaction of an aldose (VII) would yield the corresponding aldonic acid (X) and polyol (VIII), but it may well be that the cross-Cannizzaro reaction predominates during the formose reaction.

CROSS-CANNIZZARO



VII II VIII IX

CANNIZZARO



VII VIII X

The kinetics of the Cannizzaro reaction taking place during the formose reaction have been studied by Weiss and co-workers (18), but the products of this reaction were not determined, so it is not known whether the polyols were formed from the cross-Cannizzaro reaction between an aldose and formaldehyde or from the Cannizzaro reaction of an aldose.

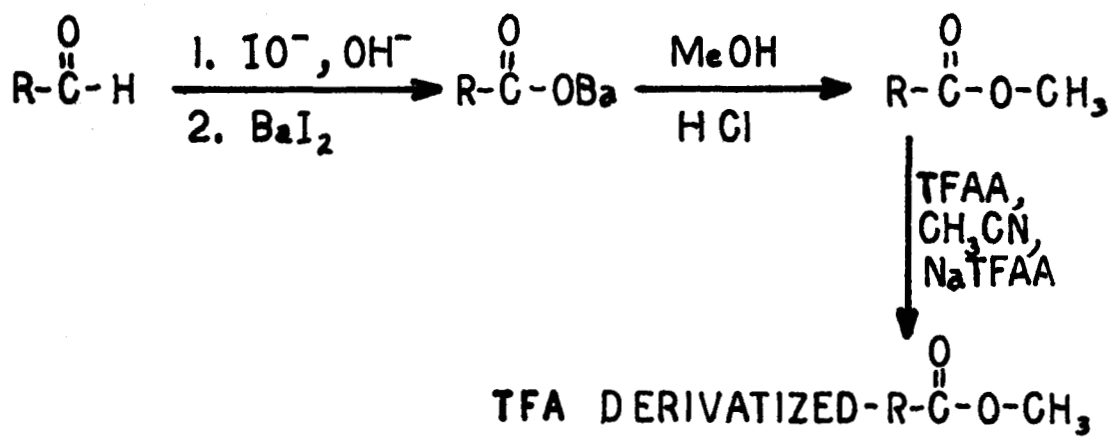
In the above experiments, a large excess of methanolic potassium hydroxide had to be avoided. In the case of LBM-6, considerable amounts of a precipitate were formed when such an excess was added. The addition of more barium iodide to the methanol solution caused more precipitate to form, and it seems most unreasonable to assume that the large amounts of precipitate formed arose from barium salts of aldonic acids; the amount of precipitate formed would indicate that the formose sugars would be almost entirely aldonic acids if this assumption were made. As long as the methanolic solution is alkaline (pH 11), there should be sufficient base present to neutralize all the aldonic acids present, and the barium salts of the aldonic acids would precipitate.

An analysis of the precipitate from LBM-6 was carried out by treating it with sodium borohydride, and the reduction product was treated with the usual TFA derivitizing solution. GLC analysis of the derivitized mixture showed about the same number of peaks found in the similar analysis of untreated LBM-6. No further attempts were made to identify this precipitated material, as it was such a complex mixture.

The precipitated material might be a complex of barium or potassium ions with the formose sugars, or it could be that co-precipitation was taking place when the concentration of ions was increased by the addition of the potassium hydroxide solution.

Aldose Oxidation Attempt.

At the same time the above work on the removal of aldonic acids from the formose sugars was being carried out, the oxidation of the aldoses in the LBM-4 formose sugars was undertaken. The method used for this was the hypoiodite oxidation of aldoses to the corresponding aldonic acid by the procedure of Moore and Link (16). Treatment of the formose sugars with iodine in methanolic potassium hydroxide solution, followed by addition of barium iodide to the reaction mixture, yielded a precipitate which was considered to be the barium salts of the aldonic acids from the oxidation. This precipitate was treated with hydrochloric acid in methanol solution so as to form the methyl esters of the aldonic acids, and when this was done, the methanol solution turned rather black. A small amount of this solution was taken to dryness to leave a black residue, and the residue was treated with TFA derivitizing solution. The expected pathway was



The GLC analysis of the resulting mixture showed the presence of an unexpectedly large number of components. Therefore, clarification of the reaction was sought.

This oxidation experiment was carried out before it was known that a solution of LBM-4, or of a formose sugar mixture, in methanol containing a large amount of potassium hydroxide would form a precipitate when barium iodide was added. So it may well be that the precipitate formed from the oxidation reaction mixture contains compounds from LBM-4 other than the barium salts of the aldonic acids. The formation of the methyl esters of aldonic acids in methanol containing hydrogen chloride is a standard procedure (19), and it seems likely that this would be so if the aldonic acids decomposed in the acidic methanol solution. On the other hand, a solution of LBM-4 turned black immediately when it was dissolved in methanol-hydrogen chloride. The oxidation of formose sugars will have to be carried out in such a manner as to avoid the precipitation of neutral components of the formose mixture with the precipitation of the barium salts of the aldonic acids.

GLC of Aldonic Acid Standards

The preparation of the TFA derivatives of the methyl esters of aldonic acids and their GLC analysis have not been reported in the literature. As we desired to use the GLC analysis of the TFA derivatives of the methyl esters of the aldonic acids obtained from the oxidation of formose sugars as an aid in the identification of the acids, we had to determine the feasibility of this method of analysis. To this end, the barium salts of gluconic, glucoheptonic, glucouronic, galactonic, ribic, and mannonic acids were treated with hydrogen chloride in methanol solution to form the corresponding methyl esters. The methanol and hydrogen chloride were removed under vacuum and the residues were treated with the usual TFA derivitizing solution. The GLC analysis of these mixtures were as follows:

TFA methyl ester	Number of peaks
galactonic	3
glycolic	0
gluconic	1
glucoheptonic	2
glucouronic	3
mannonic	3
ribic	2

Each aldonic acid, excepting glucouronic, should give but one peak for its methyl ester TFA derivative, and must give but one peak to make this analytical method feasible. Whether the spurious peaks in the above GLC analysis arise from undesired substances formed during the formation of the methyl esters, or during the TFA derivitization process is not known, and more work will have to be done to determine this. The acetates of the methyl esters of several aldonic aicds have been found to be suitable for GLC analysis of the aldonic acids (17), and the GLC analysis of the acetates of the methyl esters of the aldonic acids used above can be utilized to determine the purity of the methyl esters. If the undesired substances arise from the TFA derivitization process, then the methyl esters of the aldonic acids can be alternatively analyzed as their acetates.

Future Directions

The work which has been reported above will largely determine the work still to be carried out. The hypoiodite oxidation of formose sugars must be considered further, and hoepfully the aldoses will be removed from the formose melange by this method. Separation of the mixture of ketoses and polyols obtained from the oxidation mixture will be attempted on ion exchange resins in the bisulfite form (4), or on resins in the lithium salt form (20).

Additional effort on the preparation of the TFA derivatives of the methyl esters of the aldonic acids would be worhtwhile, as the GLC analysis of these derivatives could be a relatively easy method of analyzing for aldonic acids.

Another interesting topic would be the treatment of glucose in alkaline solution at the conditions of the formose reaction to determine the extent of gluconic acid formation from the Cannizzaro reaction of glucose. Then glucose and formaldehyde could be treated in alkaline solution under the conditions of the formose reaction in order to determine the extent of the cross-Cannizzaro reaction between glucose and formaldehyde to form sorbitol, as compared to the amount of gluconic acid from the Cannizzaro reaction.

The aldonic acids which will be isolated from the oxidation of the formose mixture could be converted to the corresponding aliphatic acids by reduction with hydrogen iodide, and the methyl esters of the aliphatic acids could be analyzed by GLC, nuclear magnetic resonance spectroscopy, and mass spectroscopy in order to identify them, as well as to determine the presence of branched chain aldoses in the formose mixtures and the distribution of 3-, 4-, 5-, 6-carbon etc., sugars present in the formose mixtures.

Once separation of the formose sugars into simpler mixtures on the basis of the different functional groups is accomplished, these latter mixtures could be separated by column chromatography according to molecular size, i.e., the 3-, 4-, 5-, 6-carbon, etc., substances from each other.

Column chromatography can be done on the formose mixtures. In this case there would be separation based on both molecular size and functional group characteristics to give mixtures simpler than the formose sugars, but containing substances of different functional groups and of varying molecular size. The components of these mixtures would still

have to be separated according to their different functional groups, and then according to molecular size. This process seems more complex than separating the components of the complete formose mixture into simpler mixtures first according to functional groups, and then according to their molecular size. Only further experimentation will show the validity of this approach to understanding the chemical composition of the formose melange.

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Appendix B

Food Utility Constant

Herbert B. Chermside and Jacob Y. Graudenz

FOOD UTILITY CONSTANT

An index of "Food Utility" has been developed which gives a useful 'handle' to the formose diet studies.

A sample of the computer output and a detailed description of the mathematical models are attached.

As is described in detail later, the two Food Utility constants are:

$$FUC1 = \frac{f(x) - f(M)}{f(C) - f(M)}$$

$$FUC2 = \frac{f(x)}{f(M)}$$

where x is treated animal data
C is "normal" control animal data
M is "minimal situation" control data
f is a function which measures weight change

Two constants were developed because, though FUC-1 is probably more precise as it uses two standardizing values, lack of minimal data often requires use of FUC-2.

The constant relates each experimental group to its appropriate "normal" control (FUC-2) or to both the "normal" control and to a "minimal situation" control (FUC-1).

Several assumptions are made regarding this model:

1) The weight change of animals represents the "usefulness" of a diet.
2) The area under a weight change curve is an appropriate measure, and is reasonably unsensitive over a 4-day period to daily fluctuations. This has not been mathematically tested.
3) The minimal case in terms of diet studies may, in our work, be represented by a period of fasting and water deprivation.

4) The minimal case for a given strain and experimental regimen may be represented by a single group of minimally treated animals, a group which is statistically rather larger than experimental groups. This is used in FUC-1 only.
5) The normal control is a group of animals allowed ad lib lab chow and tapwater. Such a control group is "treated" and measured in every experiment.
6) Standardizing strain, regimen, age, etc. can limit the variables in a feeding experiment to only those directly related to the diet fed, and further that this limitation applies with acceptable rigor to cases between different experiments having similar conditions.

With these assumptions, we believe that the FUC-1 and FUC-2 functions will produce at worst an ordinal index of "food utility" within an experiment, and that this ordinal relationship holds reasonably well between experiments. Inspection of data supports this, and even suggests that there is a continuum of "food utility" values, and the indices approximate cardinal relations on this continuum. This has not been tested mathematically.

The two indices differ. Therefore, they cannot be used interchangeably. FUC-1 is for data which includes the minimal case, FUC-2 for data without such.

The ranges and meanings of these values for each index are listed in Table 1.

Food Utility is, as yet, an imprecisely defined concept. These indices provide an explicit operational definition, but it is very limited in scope. In many respects, the situation is like that of the concept in psychology of "I.Q."; there is a generally accepted concept that it represents, but the measures are purely operational definitions and only comparable in meaning within strictly limited operational conditions.

The general concept of food utility is that a food is "good" in that it supplies all the nutrients expected of it, and has no deleterious side effects.

The two functions FUC-1 and FUC-2 should be linearly related. This relation has been demonstrated within the limits of measurement and computational accuracy.

The values for 27 determinations each of FUC-1 and FUC-2, to four places, were analyzed for linear correlation. These values constituted the results of FUC determinations on the first 9 experiments for which FUC's were computed. The coefficient of linear correlation was: $r = 0.9956$.

The deviation of this value from 1.0 (perfect correlation) may be attributed to original data measurement error (accuracy was $\pm$ 0.5 g, for readings in the 100-200 g range), cumulative rounding and truncating errors, internal representation errors in the computing process.

This high correlation is interpreted as an indication that the computational techniques are reliable, and that the range of error is acceptable.

OUTLINE OF MATH MODELS
FOOD UTILITY CONSTANTS

DAY: refers to the day number within the treatment period.

WT. CHANGE: refers to the mean change in weight for the group, for that particular day in the treatment period. For example, WT. CHANGE for DAY(1) is computed as follows:

Gp. Mean WT. CHANGE = (WT(1) - WT(0))/(DAY(1) - DAY(0))

SLOPE: represents the slope of the best fit line, as calculated via the least squares method, through the series of points representing Group mean weight change (Y) vs. day number (X). And,

$$\text{SLOPE} = \frac{\Sigma X \Sigma Y - N \Sigma XY}{(\Sigma X)^2 - N \Sigma X^2}$$

where: N = the number of input points

INTERCEPT: represents the y-intercept of the best fit line, and

$$B = \frac{\Sigma X \Sigma XY - \Sigma Y \Sigma X^2}{(\Sigma X)^2 - N \Sigma X^2}$$

CORRELATION COEFFICIENT: is the coefficient of linear correlation. The equation used is:

$$CORR = \frac{N \Sigma XY - \Sigma X \Sigma Y}{\sqrt{[N \Sigma X^2 - (\Sigma X)^2]\,[N \Sigma Y^2 - (\Sigma Y)^2]}}$$

SIGN CHANGE: there is an algorithm built into the program that sets an indicator in one of three ways:

1) NO - no sign change in mean weight changes across the treatment period.
2) YES - sign change(s) exist(s)
3) ZERO POINT - there is an interval within the treatment period in which the mean weight change is zero.

If there is a sign change and a zero point, the indicator is set by whichever occurs first within the treatment period.

Algorithm:

INDICATOR = 1,2,3
1 for NO
2 for YES
3 for ZERO POINT

The indicator is set by the following condition:

$$\frac{WC(N)}{WC(N-1)}$$

if negative, then indicator is set to 2.
if zero, then indicator is set to 3.
default is an indicator pointing to 1.

S.D. OF MEAN WT. CHANGES FOR GROUP: This is the standard deviation of the mean weight changes for the group over the measurements in the treatment period. Again, X is DAY and Y is Gp. mean WT. CHANGE:

$$S.D. = \frac{\Sigma Y^2 - (\Sigma Y)^2/M}{M - 1}$$

where: M = number of days

S.E. OF MEAN WT. CHANGES FOR GROUP: This is the standard error of the mean weight changes for the group over the entire treatment period.

$$S.E. = \frac{S.D.}{\sqrt{M}}$$

FC, FX, FM: Subscripts are as follows:

C = "normal" control animal data

X = experimental animal data

M = "minimal situation" animal data

f (written F in computer output) represents the area under a control, experimental, or minimum curve of weight change vs. day number. The area under the curve is calculated via the trapezoid rule as follows:

$$f = \Sigma\{[(WC(J) + WC(K))/2.0]\ [X(J) - X(K)]\}$$

where: J = 2....N

K = J-1

N = number of points

FUC-1: represents "Food Utility Constant-One". This constant takes the control diet as well as the minimum diet (starvation) into account in order to determine the relative food utility of the experimental diet. And,

$$FUC\text{-}1 = \frac{f(X) - f(M)}{f(C) - f(M)}$$

where f is the function described above.

FUC-2: represents "Food Utility Constant-Two". This constant is computed with respect to the control diet only in order to determine the relative food utility of the experimental diet. The algorithm is as follows:

$$\text{FUC-2} = \frac{f(X)}{f(C)}$$

where f is the function described above.

TABLE 1

VALUE AND INTERPRETATION OF FOOD UTILITY CONSTANTS

Value	Interpretation
FUC-1 > 1	Experimental diet "better than" control
FUC-1 = 0	" "same as" "
1 > FUC-1, FUC-1 > 0	" "worse than" ", but " "better than" minimal
0 > FUC-1	" "worse than" "
FUC-2 > 1	" "better than" control
FUC-2 = 1	" "same as" "
1 > FUC-2, FUC-2 > 0	" "worse than" " (but no weight loss)
0 > FUC-2	" "worse than" " (with weight loss)

45/78-7 5 PERCENT LBM-3 FORMOSE WEIGHT

5 ANIMALS IN GROUP

DAY	WT. CHANGE
1	0.50
2	2.00
3	-1.25
4	3.50

SLOPE	INTERCEPT	CORR. COEF.	SIGN CHANGE
0.574	-0.249	0.364	YES

DAILY MEAN WEIGHT CHANGES OVER TREATMENT PERIOD

SUM W.C.	N	MEAN	S.D.	S.E.
4.75	4	1.18	2.03	1.01.

FC	FX	FM
18.999	2.750	-42.890

FUC-1 = (FX-FM)/(FC-FM) = 0.7374

FUC-2 = FX/FC = 0.1447

Appendix C

Formose Liquid Feeding Protocol, 20 February 1970

Institute of Chemical Biology
University of San Francisco
San Francisco, California 94117

Formose Liquid Feeding Protocol, 20 February 1970

Male Sprague-Dawley rats from A.J. Schmidt, Berkeley, minimum of 5 per group.

Feed formose as % sol. instead of water, ad lib
Allow Lab chow ad lib at all times

Phase 1 - self control, acclimation to this lab. Approximately 1 week with ad lib chow and water. Measure weight, food and liquid consumption 3 x week.

Phase 2 - Treatment. Minimum period 5 days, maximum as needed. Ad lib chow, ad lib experimental solution. Measure weight, food and liquid consumption 3 x week or daily.

Phase 3 - Recovery. Time as needed. Ad lib chow and water. Measure weight, food and liquid consumption 3 x week.

Typical Schedule

Day	Day of Week	Age	Phase
birth	Monday	0	-
0	Monday	35	1
7	Monday	42	2
11	Friday	46	3*

* Sacrifice 2 treated animals for gross pathology.